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THE INFLUENCE OF CADMIUM IONS ON THE ADSORPTION OF PROTHROMBIN ONTO $Al(OH)_3$ AS A MEANS TO PURIFY PROTHROMBIN

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SUMMARY

1. In the presence of $CdSO_4$ (1 mM), $Al(OH)_3$ (1.3% w/v) completely adsorbs the coagulation factors VII, IX, and X from normal plasma, but factor II (prothrombin) is adsorbed for about 50% only.

2. A purification procedure for factor II is developed, using $Al(OH)_3$ adsorption in the presence of Cd^{2+} as a first step and using column chromatography only once. A 750-fold purification is obtained at a 24% yield.

3. Comparison of the prothrombin thus obtained, with prothrombin isolated by the method of Kisiel and Hanahan (Biochim. Biophys. Acta (1973) 304, 103–113) does not show significant differences in amino acid composition, N-terminal amino acid, molecular weight or immunological properties.

4. Comparison of the two prothrombin preparations in a thrombin-generating system shows that although the final yield of thrombin from a given amount of prothrombin in both preparations is the same, the initial velocity of thrombin formation from our preparation is comparable to that of native prothrombin, whereas the other preparation is converted significantly slower.

INTRODUCTION

The four coagulation factors II, VII, IX, and X adsorb together onto many inorganic powders. In the presence of Cd^{2+}, $Al(OH)_3$ adsorbs less of prothrombin than of the factors VII, IX, and X. This property can be used to purify prothrombin in a procedure employing column chromatography only once.

MATERIALS AND METHODS

The following materials were used: DEAE-Sephadex A-50 lot number 3969, and Dextran Blue 2000 from Pharmacia Fine Chemicals. Biogel P-100 polyacrylamide gel filtration material from Biorad. Acrylamide and *N,N'*-methylene bisacrylamide

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from Eastman Chemicals, New York, U.S.A. were recrystallised from chloroform and acetone, respectively. Aluminum hydroxide was obtained as a moist gel (BDH lot No. 7726301) and suspended in distilled water to a final concentration of 20% (w/v) immediately before use. Cadmium sulphate $3CdSO_4 \cdot 8H_2O$ pro analysis and all other chemicals (reagent grade) were obtained from Merck A.G., Darmstadt.

A standard plasma was obtained by mixing the plasma from 30 healthy volunteers as described in ref. 1. Due to the 1:10 dilution with the anticoagulant sodium citrate solution this plasma is defined to contain 0.9 units per ml of each of the coagulation factors.

Human plasma was obtained from blood collected on 1/10 volume of standard acid–citrate–dextrose solution in siliconized glass bottles. The blood is centrifuged for 20 min at 1500 × *g*.

The supernatant is then centrifuged for 30 min at 12 500 × *g*, which yields a plasma with less than 100 thrombocytes per mm^3.

A "euglobulin fraction" is obtained by diluting plasma 20-fold with twice distilled water at 4 °C and adjusting to pH 5.2 with 0.1 M HCl. The precipitate is collected by centrifugation (15 min, 6000 × *g*, 4 °C) and dissolved in 1/5 of the original volume of veronal acetate buffer pH 7.35.

Prothrombin was also purified according to Kisiel and Hanahan [2]. *Echis carinata* venom was obtained from Sigma. Staphylocoagulase was prepared according to ref. 5. The concentration of protein in column eluants was monitored by recording the adsorbance at 286 nm. Protein was determined according to Lowry et al. [3] with crystalline bovine serum albumin as a standard. For rapid protein measurement either a biuret method [5] was used or, with column fractions, E_{280} was measured. $E^{1\%}_{280}$ of prothrombin was taken to be 1.38 [2]. Amino acid analysis, determination of the N-terminal amino acid and molecular weight determinations by gel filtration and polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate were carried out as described in ref. 5.

Thrombin concentrations are measured by mixing 0.1 ml of sample (or, if appropriate, sample in suitable dilution) with 0.1 ml of $BaSO_4$-adsorbed bovine plasma The clotting time is recorded and compared to the clotting time obtained with dilutions of a standard preparation of thrombin. The thrombin generation experiments were carried out as described in ref. 12. One-stage assays for factors V, VII, and X were carried out and evaluated as described in ref. 6. The two-stage assay for factor II was carried out and evaluated as described in ref. 7. Factor IX was estimated according to ref. 1. Determination of the prothrombin as the staphylocoagulase-reacting factor was carried out as described in ref. 8.

Determination of prothrombin by means of *Echis carinata* venom was carried out in the same way, substituting a suitable concentration of the venom for staphylocoagulase. Tosyl arginine methyl esterase activity was assessed as described in ref. 9.

RESULTS

As can be seen from Fig. 1, the presence of $CdSO_4$ specifically prevents the adsorption of prothrombin from normal plasma onto 2% (w/v) $Al(OH)_3$, $CdCl_2$ and cadmium acetate show the same effect. Comparable results were obtained at other $Al(OH)_3$ concentrations. Mn^{2+}, Ni^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+} were found not to influence

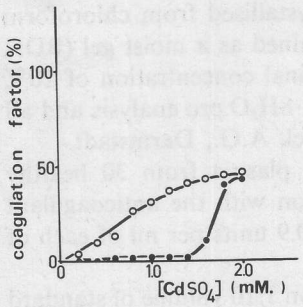

Fig. 1. Influence of Cd^{2+} on the adsorption of coagulation factors onto $Al(OH)_3$. $CdSO_4$ was added to normal plasma to the final concentration indicated. 2% (w/v) of $Al(OH)_3$ was added, incubated for 10 min and separated by centrifugation. The concentration of coagulation factors in the supernatant was determined. ○—○, factor II; ●—●, factor X. The values for the factors VII and IX were 0 at Cd^{2+} concentrations <1.4 mM and differed less than 5% from those for factor X, at higher concentrations; they are not represented.

the adsorption. We further purified the prothrombin not adsorbing onto $Al(OH)_3$ in the presence of 1.0 mM Cd^{2+}.

Purification procedure

The entire procedure is carried out at 4 °C. To the plasma are added successively 1/100 volume of a 0.1 M $CdSO_4$ solution and 1/15 volume of $Al(OH)_3$ in the form of a 20% (w/v) moist gel. After stirring for 10 min the $Al(OH)_3$ is removed by centrifugation for 15 min at 6000 × *g*. The Cd^{2+} from the supernatant (supernatant a, Table I) is removed by adding 3 g solid sodium oxalate per 100 ml, and centrifugation (15 min, 6000 × *g*) of the cadmium oxalate precipitate.

From the supernatant (supernatant b, Table I) prothrombin is adsorbed onto 20% (w/v) $Al(OH)_3$ which is sedimented as above. The $Al(OH)_3$ sediment is washed first with 1/5 volume 0.1 M EDTA (pH 8.0) and then with 1/5 volume 0.15 M NaCl. The proteins adsorbed are eluted with 1/20 volume 0.25 M sodium–potassium phosphate buffer (pH 8.0) and the $Al(OH)_3$ is removed. The eluate is dialysed overnight against 0.1 M NaCl in 0.01 M sodium–potassium phosphate buffer pH 6.8 and applied to a 9 × 1 cm column containing DEAE-Sephadex A-50 previously equilibrated with 0.1 M NaCl in 0.01 M sodium–potassium phosphate buffer pH 6.8; 30 ml of

TABLE I

PURIFICATION OF HUMAN PLASMA FACTOR II

The figures are the means from 12 batches.

Fraction	Vol. (ml)	Activity (units/ml)	Protein concentration (mg/ml)	Spec. act. (units/mg)	Recovery (%)	Purification (fold)
Plasma	500	0.83	68.8	0.0121	100	1.00
Supernatant a	535	0.44	67.0	0.0066	57	0.54
Supernatant b	535	0.42	67.0	0.0063	54	0.52
$Al(OH)_3$ eluant	25	5.98	5.90	1.014	36	84
Sephadex A-50	44	2.26	0.25	9.04	24	749

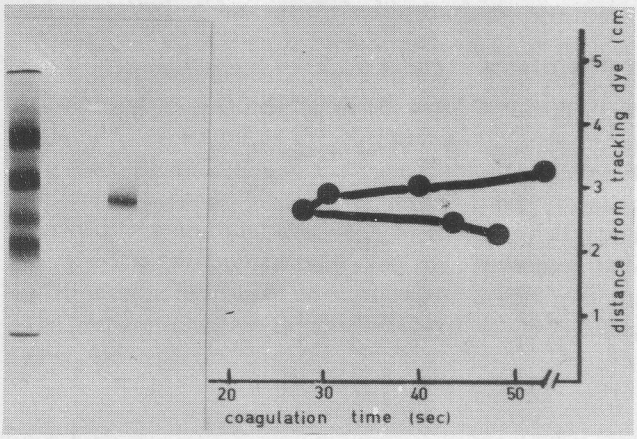

Fig. 2. Polyacrylamide gel electrophoresis of human factor II. Left hand gel: sample before chromatography; right hand gel: sample after chromatography. The graph gives the coagulation times in a factor II estimation as found with fragmented 2-mm segments of a gel obtained under the same conditions as B.

this buffer is then followed by a linear gradient in the same buffer of 0.0–0.6 M in NaCl (2 times 100 ml). Prothrombin elutes as a single peak at about 0.43 M NaCl. The fractions containing more than 30% of the original factor II activity are pooled.

Properties of the purified material

No factor VII, IX, or X activity was detectable in the final preparations; in our assay systems (b) the limit of detectability is 0.0025 units/ml of the factors VII, X, or IX in the presence of 1 unit/ml factor II.

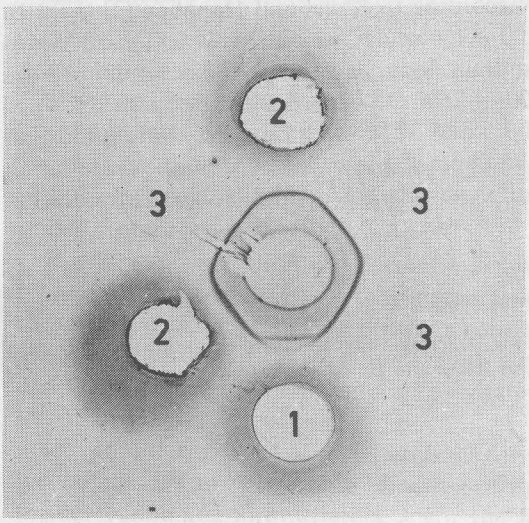

Fig. 3. Immuno diffusion pattern with an antibody against prothrombin. The centre well contains the antibody. The other wells contain human serum (1), human plasma (2) and purified prothrombin (3). Two types of the latter are tested, upper wells: preparation as described in this article; lower well: preparation according to Kisiel and Hanahan [2].

TABLE II

AMINO ACID COMPOSITION OF HUMAN FACTOR II

The figures give g amino acid per 100 g protein. Mean of four different preparations.

Amino acid	Lanchantin [10]	Hanahan [2]	Present research
Lysine	4.81	5.19	4.85
Histidine	1.78	1.91	1.98
Arginine	7.94	8.34	8.18
Aspartic acid	9.14	9.85	9.89
Threonine	5.52	4.90	5.21
Serine	4.67	4.36	4.25
Glutamic acid	12.72	13.17	13.36
Proline	4.28	4.28	4.50
Glycine	3.52	3.83	3.75
Alanine	3.48	3.75	3.61
Cystine (half)	3.35	2.51	3.84
Valine	4.15	4.53	4.53
Methionine	1.30	1.29	1.38
Isoleucine	3.42	3.30	3.18
Leucine	6.11	6.52	6.48
Tyrosine	4.90	4.31	4.95
Phenylalanine	4.42	5.07	3.80
Tryptophan*	5.34	3.28	3.09
Totals	90.85	90.39	90.83

* Determined according to Scoffone [16].

The isolated protein shows a single band on polyacrylamide-gel electrophoresis (Fig. 2). An antibody raised against this preparation showed one precipitation arc with normal human plasma continuous with both our preparation of purified prothrombin and a preparation prepared according to Kisiel and Hanahan [2] (Fig. 3).

By gel filtration on Biogel P-100 a molecular weight of 74 000 $\pm$ 2000 was found (means of 15 experiments). On sodium dodecylsulphate-gel electrophoresis the molecular weight appeared to be 72 000 $\pm$ 2500 (15 experiments) (see also ref. 9).

Upon incubation for 4 h at pH 7.5 and 37 °C both our preparation and that prepared according to Kisiel and Hanahan were stable as judged from the degradation products detectable in sodium dodecylsulphate-gel electrophoresis. Upon the addition

TABLE III

SPECIFIC ACTIVITY OF PURIFIED PROTHROMBIN

The figures give specific activities in units per mg. A, preparation as described in this article (means of 12 experiments); B, preparation according to Kisiel and Hanahan [2] (means of 2 batches).

Method	Preparation	
	A	B
One-stage	9.04	5.34
Two-stage	9.06	9.34
Staphylocoagulase	9.07	9.02
Echis carinata	9.01	8.98

of 1 unit/ml of human thrombin both preparations broke down within 30 min to 50 000 and 23 000 mol. wt subunits, which is in accordance with the literature [17].

The amino acid composition is not essentially different from that of the human prothrombin purified by Kisiel and Hanahan [2] and Lanchantin [10] (Table II). Alanine was found as the N-terminal amino acid. The final preparation contained no traceable amounts of Cd^{2+}. In Table III the specific activities obtained with four different methods of assaying prothrombin are compared for the preparation described here and that described by Kisiel and Hanahan [2].

The two preparations were also compared as to the velocity of thrombin formation by a fixed high concentration of prothrombinase [12]. The concentrations of the preparations were adjusted so as to yield eventually the same amount of thrombin (Fig. 4).

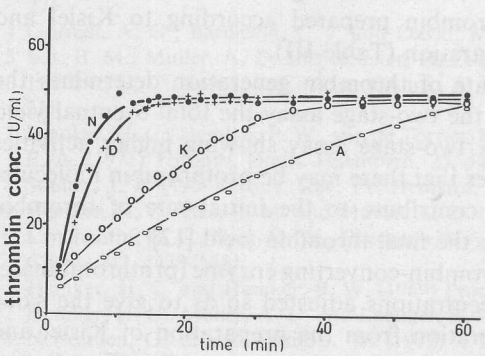

Fig. 4. Velocity of thrombin generation from various preparations of prothrombin. At zero time a fixed high amount of prothrombinase is added to a prothrombin preparation. The incubation is done at 37 °C. At 2 min intervals 0.1-ml aliquots of the incubation mixture are assayed for their thrombin content. N, euglobulin preparation from normal plasma; D, prothrombin preparation as described in this article; K, prothrombin preparation prepared according to ref. 2; A, euglobulin preparation from patients under deep oral anticoagulation. The final thrombin yield was assessed in preliminary experiments. The preparations were diluted so as to gain the same final thrombin concentration in this experiment.

Also included in this experiment is the thrombin generation from an acid-precipitated euglobulin fraction from the plasma of normal subjects and of patients under deep oral anticoagulation.

Acid precipitation was necessary as it is compulsory in this type of experiment to remove antithrombin 3. These preparations were included in order to compare the velocity of thrombin generation from the purified preparations with that of normal prothrombin that has undergone a minimum of manipulations on the one hand, and with the protein induced by vitamin K absence analogous to factor II on the other [12].

In order to check the preparations for traces of thrombin, the preparation was incubated with an "euglobulin" fraction of $BaSO_4$-adsorbed normal oxalated plasma neither containing antithrombin 3 nor the factors II, VII, IX, and X, but rich in factor V (150%) and factor VIII (185%); no activation of these factors could be observed.

In a solution containing 200 μg/ml of prothrombin and $5 \cdot 10^{-3}$ M tosyl–arginine methyl ester, less than $5 \cdot 10^{-8}$ g equiv. was split in 10 min. Under the same circumstances 1 μg of thrombin (2.5 N.I.H. units) per ml will split $1.5 \cdot 10^{-6}$ g equiv. of the ester.

DISCUSSION

The prothrombin that does not adsorb to $Al(OH)_3$ in the presence of 1 mM $CdSO_4$ could not be distinguished from the prothrombin described by Kisiel and Hanahan [2] on the basis of amino acid composition, N-terminal amino acid, immunological properties or molecular weight. There are certain differences between the two preparations, however, in the kinetics of thrombin generation.

We confirmed the discrepancy between the one-stage and the two-stage determination of factor II activity in prothrombin prepared according to Kisiel and Hanahan, but did not find it in our preparation (Table III).

In a one-stage assay the initial rate of thrombin generation determines the outcome of the estimate [11] whereas in the two-stage assay the total eventual yield of thrombin determines the result [7]. A two-stage assay showing higher activities than a one-stage assay, therefore, indicates that there may be prothrombin molecules present that do not (or only partially) contribute to the initial rate of thrombin generation whereas they do contribute to the final thrombin yield [12].

With a fixed high amount of prothrombin-converting enzyme (prothrombinase) present, and with the prothrombin concentrations adjusted so as to give the same final thrombin yield, the thrombin generation from the preparation of Kisiel and Hanahan indeed is significantly slower than that from our preparation (Fig. 4).

We compared the kinetic properties of the purified preparations to those of comparatively unmanipulated prothrombin and of the protein induced by vitamin K absence analogous to factor II (PIVKA-II) [13, 14]. Like the prothrombin of Kisiel and Hanahan, PIVKA-II shows a discrepancy between the one-stage and the two-stage assay [12]. Also the velocity of thrombin generation from PIVKA-II is much slower than that from normal prothrombin (ref. 12; Fig. 4). It is thought that the difference between PIVKA-II and normal prothrombin lies in the fact that in normal prothrombin several glutamic acid residues have been converted into γ-glutamyl glutamic acid residues by a vitamin K-dependent system [15]. In PIVKA-II the γ-carboxy glutamic acid residues are absent, which makes this protein a bad substrate for prothrombinase.

It is tempting to attribute the observed kinetic properties of the Kisiel and Hanahan prothrombin preparation to the loss of one or more γ-carboxyl groups from the prothrombin molecule during the purification procedure. Alternatively, one might suspect the rapid generation of thrombin in our preparation to be caused by trace amounts of thrombin acting upon factor V in the prothrombinase preparation. The complete absence of any activation of the factors V and VIII by our preparation and the absence of an autodigestion pattern as would be obtained if thrombin were present makes this explanation much less likely. From the Tos-Arg-OMe esterase activity of the preparation it can be concluded that less than 0.05 μg thrombin per 100 μg of prothrombin is present in our preparation.

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